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Conference Report

NIST WORKSHOP ON LUMINESCENCE STANDARDS FOR CHEMICAL ANALYSIS Gaithersburg, MD September 8-9, 1999

Report prepared by

Paul C. DeRose

Analytical Chemistry Division,
Chemical Science and Technology Laboratory,
National Institute of Standards and Technology,
Gaithersburg, MD 20899-8394

paul.derose@nist.gov

Available online: http://www.nist.gov/jres

1. Introduction

About 40 scientists from the clinical and biotechnology communities, instrument and standards vendors, federal agencies, academia and five national metrology institutes (NMIs) attended this 2 day workshop, focusing on standards for fluorescence-based chemical analysis, held at the National Institute of Standards and Technology (NIST) in Gaithersburg, MD. The workshop was jointly sponsored by the Analytical Chemistry and Biotechnology Divisions of the Chemical Science and Technology Laboratory (CSTL) and the Optical Technology Division of the Physics Laboratory (PL) at NIST. Each day's agenda included nine speakers followed by open discussion. The goals of the workshop were to assess the importance and needs for fluorescence standards as well as to appraise new materials and technologies that might be candidates for such standards.

Luminescence measurements have become the methods of choice for new clinical and biochemical analyses due to their extraordinary selectivity and high sensitivity. Reporter gene and other biomolecular probe technologies now make it possible to label biochemical entities with high specificity and to detect the marked species with near single molecule sensitivity in extremely heterogeneous environments, such as living cells. Luminescence measurements provide the analytical grease for many current fast track technologies, including genetic engineering and high throughput screening, which would not be taking place without luminescence measurements. For many years, luminescence has been used for detection of biological and drug active sites in qualitative screening techniques, which has yielded a long list of site-specific chemical leads. There exists a growing desire now to assess the quality of leads quantitatively, which requires standards to calibrate luminescence measurement instruments and to validate the analytical methods themselves. In the highly regulated pharmaceutical and clinical industries such standards are needed to satisfy the quality assurance and validation requirements of national health certifiers and regulators, such as the U.S. Food and Drug Administration (FDA).

The attendance of representatives from other NMIs, including the Federal Institute for Materials Research and Testing−Germany (BAM), the National Physical Laboratory−United Kingdom (NPL), the National Research Council−Canada (NRC) and the Physikalisch-Technische Bundesanstalt−Germany (PTB), illustrated the international interest in fluorescence standards development. The increasing importance of drug and food testing is making the needs for fluorescence standards components of international trade issues and sparking collaborations and intercomparison activities among the NMIs.

Of the eleven speakers from outside of NIST, there were four from industry, four from academia, two from other NMIs and one from the Centers for Disease Control (CDC). Presentations included a discussion of needs for fluorescence standards for pharmaceutical screening, instrument validation and cytometry, and reports on a variety of new fluorescence standards candidate materials, including nanocrystals. The workshop emphasized the need for fluorescence standards in the areas of fluorescence spectrometer calibration and clinical analysis.

2. Presentation Summaries

The Workshop on Luminescence Standards for Chemical Analysis was conducted over two days with each day divided into three technical sessions: "Fluorescence Spectrometry and Standards," "Standards Candidates," and "Biotechnological and Clinical Applications of Luminescence and Related Standards. " Welcoming remarks were made by W. F. Koch, Deputy Director, CSTL, followed by an orientation by P. C. DeRose, research chemist and principal technical organizer. Each technical session was made up of half-hour talks by invited speakers, each talk followed by 15 minutes of questions and discussion. A 1 hour open discussion closed out each day, during which a search for consensus on those fluorescence standards that are most needed was explored. A detailed summary of each speaker's presentation is given in the subsections that follow.

2.1 Luminescence Research at NIST

Rance A. Velapoldi—NIST (retired)

The luminescence research performed at NIST over the last 35 years has included 1) determining the suitability of materials, such as inorganic ions in polymers and glasses, and organic species in solution, as standards; 2) taking quantitative measurements of species in the gas, solid and solution phases, for example, determining the solubility and distribution coefficients of polyaromatic hydrocarbons (PAHs) in aqueous solutions; and 3) developing derivative or information standards by, for instance, using the chromium lines in ruby to measure the pressure in a diamond anvil cell or measuring the quantum yield of the ferrioxalate actinometer.

The spectral emissivities and relative quantum yields of "television" phosphors were determined in the 1960s. [1]. Further research on these inorganic phosphors [2], sintered in polytetrafluoroethylene (PTFE), lead to the certification of SRM 1931, Fluorescence Emission Standards for the Visible Region, in 1989 by A. Thompson and K. L. Eckerle. SRM 1931, consisting of four front-surface-fluorescing, opaque solids mounted in cuvette holders, is a spectral emissivity standard that is no longer commercially available.

In the early 1970s, standards were determined to be needed in fluorescence spectrometry for wavelength, spectral emissivity, quantum yield, quantum counters, lifetime and polarization. The characteristics deemed desirable in these materials were stability, ease of purification, minimal spectral overlap of excitation and emission spectra, minimal oxygen quenching, high quantum yield, and isotropic emission. The clinical chemistry community desired fluorescence standards in aqueous solution and the National Institute of General Medical Sciences (NIGMS), one of the National Institutes of Health (NIH), partially funded this research at NIST. A unique spectroradiometer was designed and built [3,4] for performing high-precision luminescence research and making the highly precise and accurate measurements that are critical for certifying national standards. This lead to the certification of the first fluorescence standard issued by NBS, SRM 936 (Quinine Sulfate Dihydrate), in 1979 by R. A. Velapoldi and K. D. Mielenz [3]. SRM 936 is a spectral emissivity standard supplied as highly purified quinine sulfate dihydrate powder that is to be dissolved in a perchloric acid solution. Other organic compounds that were studied and compared with quinine sulfate were fluorescein, rhodamine B, cresyl violet, 3-aminophthalimide, 2-aminopyridine and β-carboline.

During the 1980s, the luminescence properties of more than 20 transition and rare-earth metal ions in glasses were also characterized using macro- and microspectrofluorimetry [5]. For example, the spectral emission range of $(UO_2)^{2+}$ is similar to that of fluorescein and the properties of glass beads doped with $(UO_2)^{2+}$ were compared to beads labeled with fluorescein isothiocyanate (FITC), a commonly-used fluorescein derivative. The inorganic ion-doped glasses were at least three orders of magnitude more photostable than the organic labeled materials. The effect of excitation cubes in microspectrofluorimeters was also reported.

2.2 Fluorescence Standards: What is Needed; an Introduction to Instruments, Samples and Limits

Jeffrey Taylor—Perkin Elmer

Fluorescence spectroscopy is one of the fastest growing methodologies in the life science, biotechnology and pharmaceutical marketplace. This growth has prompted a proliferation of novel instrument designs. The demand for task-specific fluorometers, such as well plate scanners, has increased by 30 % in the past year, while that for general-purpose fluorometers has not

changed. Challenges relating to the specification of appropriate Standard Reference Materials for this diverse cross-section of instrumentation must be addressed. The growth of fluorescence techniques has been driven by the production of fluorescent probes with sensitivity and selectivity rivaling that of radioactive labels without the associated handling and disposal problems.

Common viewing geometries for fluorometers include (1) 90° viewing, relative to the incident excitation beam, for cuvettes and cuvette-shaped transparent solids; (2) front surface viewing at less than 90° relative to the sample normal, typically 60°, of opaque solids or turbid liquids; (3) fiber optic viewing at 0°, emission is collected on the same side from which it is being illuminated, with a bifurcated fiber or fiber bundle. Excitation and emission wavelength selection is accomplished in fluorometers using a bandpass filter or a monochromator with a diffraction grating.

Intrinsic characteristics of fluorescent species must be considered when designing experimental methods or assays. These include (1) the nature of the Beer-Lambert law, resulting in non-linearity of fluorescence intensity at high concentration due to non-linear absorption; (2) the environmental dependence of the wavelength and intensity of fluorescence; and (3) the lack of photometric accuracy standards for fluorescence, making calibration and intercomparison of results difficult.

The challenges presented by different geometries and sample formats must be realized to achieve satisfactory reproducibility and accuracy of emission spectra. For a 90° viewing geometry, dilute samples should be used to prevent absorption by the sample of a significant fraction of (1) the excitation light before it reaches the detection region in the sample and (2) the emission before it exits the sample; these are known as inner filter effects. Luminescence from opaque or translucent solids is observed using a front surface geometry. Solid medium luminescence is subject to high background, due to greater scattering, and pronounced non-linearity of intensity versus concentration, due to the absorptivity of the sample medium. Detection at the specular angle of reflection is not conducive to being sensitive to front surface fluorescence. If a sample does not luminesce, a signal is often still detected from diffuse reflection of the source, so it is important to be able to recognize the source spectrum.

Many task-specific fluorometers, such as plate and chip readers, use bandpass filters for wavelength selection. These instruments are not sensitive to fluorophores with small Stokes shifts, due to overlap or cross talk between excitation and emission spectra. Micro-well plates are sold in different sizes and colors, and opacity, which can affect their performance. Opaque plates must be read from the top, and if the plate's material is not completely opaque, light piping and cross talk between wells can occur. Front surface plate measurements, such as those used to detect fluorescence from cells, are read from the bottom. Fluorescence intensity is proportional to the amount of fluorescent species in a well for a plate reader, whereas the intensity for a standard fluorometer is proportional to concentration. Therefore, to make a calibration curve for intensity, it is necessary to deliver specific amounts to a standard well plate, and it is not sufficient to just specify concentration. White plates or black plates are both commonly used. White plates give higher signal levels, but also higher background noise from light scattering. Black plates give minimal noise levels, but only modest signal levels as the excitation and emission light are not reflected by the walls.

2.3 A New Concept for Liquid Fluorescence Reference Materials

Christoph Saal—Merck KGaA, Darmstadt

Most users of fluorescence spectrometers need standards for periodic instrument validations, at which time, the tested parameters of the instrument must match the certified values within instrument and standard uncertainties. Such standards should be easy to use, of a high quality and reliability and traceable to an established primary standard or natural constant. The parameters that have to be checked to guarantee correct results from a fluorescence spectrometer include linearity of fluorescence intensity, long-term stability, wavelength, spectral resolution and, when applicable, lifetime and/or polarization.

Linearity and long-term stability of fluorescence intensity can be monitored using ASTM Designation E578-83, Standard Test Method for Linearity of Fluorescence Measuring Systems. This method uses several concentrations of quinine sulfate dihydrate in dilute H_2SO_4(aq) to determine intensity as a function of concentration. Quinine sulfate dihydrate is sold in powder form by NIST as SRM 936a. Merck KGaA will sell a set of ampouled solutions for single use with concentrations specified in ASTM Designation E578-83. Specifications and use of the test set will be described in the accompanying certificate and instructions for use.

A comparison of the excitation and emission spectra of quinine, quinine sulfate dihydrate and dihydroquinine dissolved in aqueous sulfuric acid show all three to be nearly indistinguishable from one another. This is important because dihydroquinine is an impurity found in commercially available quinine sulfate

dihydrate and quinine. Considering the similarity between the fluorophores in solutions of quinine and quinine sulfate dihydrate in aqueous sulfuric acid, the correspondence of their fluorescence spectra is expected. The certificate for SRM936a, a relative spectral emissivity standard, specifies perchloric acid to be used to make up the standard solution, whereas ASTM Designation E578-83 specifies sulfuric acid to be used. It is observed here that dissolving quinine sulfate dihydrate in either sulfuric acid or perchloric acid produces very similar spectra.

For those that follow the E578-83 test method, it should be noted that 10^{-3} μg/ml to 10^{4} μg/ml quinine sulfate dihydrate solutions are prescribed for the linearity test, but it is observed here that inner filter effects become significant at concentrations at or above 100 μg/ml.

2.4 New Fluorescence Applications in Cell Biology and Pharmaceutical Screening Need New Standards

Roger Y. Tsien—University of California, San Diego

Why is fluorescence becoming so important in pharmaceutical screening? Detection of fluorescence requires fewer probe molecules than other readout modes, making ultraminiaturization possible. Assays can be performed in a homogeneous format so that a washing step is not required. A wide variety of fluorophores, including genetically encodable ones like green fluorescent protein (GFP), are commercially available. Fluorescence is also well suited to a wide variety of readout mechanisms (intensity, wavelength, etc.) and observation formats (standard fluorometer, microscope, plate reader, flow cytometer, gel scanner, hybridization chip).

Major types of dynamic fluorescence assays in cell biology and pharmaceutical screening include (1) ion indicators [6], which change intensity or excitation or emission wavelengths in response to Ca^{2+} and other physiologically important ions; (2) GFP fusions to proteins of interest[7,8,9]; (3) reporter gene assays to measure gene expression [10]; (4) voltage-dependent fluorescence resonance energy transfer (FRET) to assay ion channel function [11]; (5) time-resolved luminescence resonance energy transfer from lanthanide chelates to red dyes or phycobiliproteins [12]; (6) fluorescence polarization assays [12]; (7) nucleic acid hybridization and polymerase chain reaction (PCR) readouts, using FRET between labeled nucleotides.

Industry-wide fluorescence standards for fluorescent assays do not exist, but are greatly needed. Solutions of organic dyes are most commonly used for laboratory standards. They are convenient and versatile, but photobleach relatively quickly. Solid inorganic standards are more stable, but need to mimic real-life wavelengths, concentrations and formats to achieve widespread acceptance. Fluorescence intensity is most commonly standardized within individual laboratories using fluorescein solutions with known concentrations. As much as a 10 % uncertainty may arise due to the varying purity of available fluorescein. When comparisons are made between high throughput screening (HTS) laboratories, differences of two to three orders of magnitude are often observed in the quantitative results of comparable assays. Thus standardized protocols are even more critically needed than standardized materials. It is proposed here that fluorescent beads with defined bleachability may be an effective way to quantify both the excitation intensity and the detection efficiency of a particular fluorometer.

FRET has been demonstrated to be a very effective detection mechanism for assays, due to its sensitivity to molecular proximity and its detectability as an intensity ratio of two different colors of fluorescence. Such ratios are advantageous, because they are independent of sample volume, excitation intensity and probe concentration. FRET can be used to monitor the cleavage of proteins at protease sites [9] as well as reversible protein-protein interactions. Gene expression can be monitored with very high sensitivity in live unpermeabilized mammalian cells by using bacterial β-lactamase as a reporter enzyme, which cleaves dye-labeled antibiotics and thereby disrupts FRET between the two halves [10]. Changes in cell membrane potentials have also been measured by FRET between donors bound to the extracellular surface and fluorescent acceptor anions that translocate across the membrane in response to depolarization [11].

Aurora Biosciences Corp. is working to meet the challenges of ultra-high-throughput pharmaceutical screening. Aurora designs and performs fluorescence-based assays of targets and signaling mechanisms both in vitro and in mammalian cells. Such assays can be miniaturized to 1 μl to 2 μl volumes in plates with 3456 wells, using automated sample handling, high-speed fluorescence readers, and integrated data management software, enabling very large numbers of assays to be performed much faster and with much smaller samples than previously required [13].

2.5 Fluorescence Lifetime Standards

Zygmunt Gryczynski—University of Maryland, Baltimore

Candidate fluorescence lifetime standards for picosecond, nanosecond and microsecond timescales are now available. The areas of chemistry, biochemistry

and biophysics in which they are most needed include basic physical chemistry, chemical sensing, clinical sensing and diagnostics, microscopy and flow cytometry. There are two types of lifetime measuring instruments used, (1) time domain and (2) frequency domain. These involve (1) a single excitation pulse followed by time-resolved emission and (2) an intensity-modulated excitation that is also frequency modulated, where the lifetime is determined from the intensity modulation ratio m, the phase angle ϕ, and the frequency of modulation ω [14].

Fluorescence lifetime standards are needed, due to the complexity of instruments and analyses involved and the errors that can subsequently result. Systematic errors can be introduced into lifetime measurements by a number of factors. These include sample concentration, sample geometry and polarization of light (excitation and emission). For picosecond or shorter lifetimes, length of cuvette and optical delays on optically active elements are also significant factors. These errors make it particularly difficult to resolve lifetimes for samples with multiexponential decays, which includes most samples of interest, where lifetime parameters are often distorted and have large uncertainties. To fit a fluorescence decay profile that contains three components, much has to be known about the system prior to the lifetime analysis, such as the identity of the three components.

Useful fluorescence lifetime standards should (1) display a single exponential decay, (2) be effective at desired excitation and emission wavelengths and timescales, (3) be made from components that are readily available at high purity, (4) be able to be prepared easily and reproducibly, and (5) possess large Stokes shifts. Stilbene and styrene derivatives possess desirable characteristics for tens to hundreds of picoseconds (ps) lifetime standards. 4-dimethylamino-trans-stilbene derivatives are under development for less than 20 ps fluorescence lifetime standards. Some organic dye solutions are good candidates in the 1 nanosecond (ns) to 10 ns range, such as 2,5-diphenyl-1,3,4-oxadiazole (PPD) in ethanol (τ = 1.24 ns, λ_{EM} = 315 nm to 390 nm) and Rhodamine B in water (τ = 1.58 ns, λ_{EM} = 575 nm to 620 nm). Ru and Re metal complexes in solution and polymer films are candidates on the 100 ns to microsecond (μs) timescale, and rare-earth complexes could be useful on the millisecond (ms) timescale.

Presently, fluorescence lifetime standards for multiphoton excitation as well as vacuum UV emission are being studied. Multi-photon excitation has the advantages of red/NIR excitation, minimal photo-damage, localized excitation and confocal imaging capabilities. Fluorophores that emit in the vacuum UV are called "extremofluors" and include common solvents such as cyclohexane.

2.6 The Future of Fluorescence Standards

Edward J. A. Pope—MATECH

MATECH was founded in 1989. At present, their primary focus is manufacturing fluorescence standards. The company is registered with the FDA and licensed by the state of California as a "medical device manufacturer." Their TR-series (top reading) 96 well plate fluorescent reference materials are distributed and marketed by Precision Dynamics Corp. and listed with the FDA as a Class I medical device [510(k) exempt]. The reference plates come in three colors: blue (TR-418), green (TR-517) and red (TR-613). The number in each plate designation is the wavelength in nm of maximum fluorescence intensity and can be used for the calibration of instruments for wavelength and intensity. Top reading 384 well plate and bottom reading 96 and 384 well plate standards are currently being developed, along with a serial dilution standard scheduled for release in late 2000. In addition, MATECH is an OEM manufacturer and supplier of custom fluorescent standards for several medical instrument manufacturers.

Fluorescent standards can be categorized into groups, based upon the fluorophore and host medium of which the standard is comprised. Fluorophore-host groups include organic dye—solution, organic dye—polymer, organic dye—inorganic solid, inorganic ion—polymer, inorganic ion—inorganic solid. Organic dyes photobleach in a relatively short time. A polymer host may also fluoresce and be photobleached by the fluorophore-excitation source, causing changes in the detected fluorescence intensity with time. Inorganic ions—inorganic solid is the fluorophore-host grouping that offers the best photostability. The TR-series standards fall into this group; each standard is a metal ion fluorophore in an inorganic glass host. The fluorescent ions used are Ce^{3+}, U^{6+} and a combination of Eu^{3+} and U^{6+} in TR-418, TR-517 and TR-613, respectively. Severe photobleaching data, taken during weeks of exposure to high intensity 254 nm, 365 nm, and 470 nm light, are compared for TR-517, rhodamine B in poly(-methylmethacrylate) (PMMA) and fluorescein in PMMA. Both organic dyes show significant photobleaching, whereas TR-517 shows little, if any, effect. Thermal and humidity cycling data, taken over a comparable time period, show a gradual decrease in fluorescence intensity with time for the organic dyes. Again, there is little, if any, effect on the fluorescence intensity of TR-517.

MATECH presently validates their master standards for the TR-series using SRM 936a, which covers the 375 nm to 675 nm spectral region with increasing uncertainty toward the extremes of the spectral range. This validation works well for the blue master standard, not as well for the green and not at all for the red. The master standards are then used for quality control testing of the TR-series standards. What they hope becomes available in the future is a NIST "universal" standard that would cover the entire visible spectrum. This type of standard would enable a complete validation of the master standards, which would make both the master and TR-series standards NIST traceable. It may be possible for MATECH to make such a material for NIST by doping glass with several fluorescent metal ions. NIST could then certify the material as an SRM and sell it to secondary standards makers, who would produce NIST traceable standards for different fluorescence detection formats.

2.7 Fluorocarbon Matrix Based Luminescent Reference Materials

Art Springsteen—Avian Technologies

Stable, front-surface luminescent reference materials may be produced by incorporating stable inorganic luminescent materials, both fluorophores and phosphors, into a fluorocarbon matrix. This patented procedure has been used by Labsphere for a number of years to produce a blue-white fluorescent material that is used as a standard in the pulp and paper industry due to its spectral similarity to the optical brighteners used in paper. The fluorocarbon matrix, called Spectralon, is made of sintered, low-density polytetrafluoroethylene (PTFE) and used as a diffuse reflectance standard. The advantages of Spectralon as a matrix for fluorophores include that it is inert, identical throughout, stable to UV light, non-spectrally selective and can be combined with multiple luminescent materials in the same matrix.

The disadvantages of Spectralon include (1) it can only be used for front-surface measurements; (2) it is difficult to make homogenous, resulting in a speckled appearance at low concentrations; (3) only inorganic fluorophores that can withstand the high temperature production process can be incorporated into the matrix; (4) compatible fluorophores are primarily UV-activated; (5) inorganic fluorophores usually cannot duplicate the small Stokes shifts of many organic fluorophores.

Several different Spectralon luminescent materials have been produced, emitting blue-white, blue, green, orange and red light [15]. Except for the red and yellow fluorophores, all are currently available. SFS-461 contains a blue-white fluorophore and is the above-mentioned standard in the pulp and paper industry. Dilution studies show that the luminescence intensity of these materials is not linear with fluorophore concentration, unless carbon black is added to the matrix. By adding carbon black, an absorber, the amount of light reaching the fluorophore can be controlled.

Several points should be made concerning fluorescence standards. Different users are measuring different fluorescence properties using different instrument formats, so a single standard will not satisfy everyone. Intensity standards are particularly important, but are not interchangeable between instruments. A large range of instrument bandpasses must be considered, when certifying a standard, to make it useful for a variety of fluorometers.

2.8 NIST Standard Reference Materials Program

Joylene W. L. Thomas—NIST

The SRM Program is the central point at NIST for distribution and marketing of all reference materials, services and related activities. In this capacity, the Program (1) performs continuous analysis and needs assessment of national reference materials and requirements; (2) establishes and promotes uniform criteria for the development and certification of reference materials; (3) performs technical activities related to the preparation, packaging and distribution of SRMs; (4) markets SRMs using a number of tools including the on-line and printed SRM catalog and a variety of published documents and articles such as journal articles, brochures and newsletters; (5) provides independent review of the SRM value assignment process; (6) establishes, jointly with NIST technical divisions, vertical traceability links with the U.S. secondary reference materials producers; (7) provides customer support to purchasers and users of SRMs; and (8) provides official NIST representation on national and international standards committees.

2.9 Approaches to Luminescence Standards

Ute Resch-Genger—BAM, Berlin, Germany

Steady-state and time-resolved fluorescence studies for the development of certified reference materials are performed at the Federal Institute for Materials Research and Testing (BAM) in the Department of Analytical Chemistry in cooperation with the Temperature Radiation group at Physikalisch-Technische Bundesanstalt (PTB). The latter group establishes and maintains Germany's primary standards for both the spectral radiance scale, using high temperature black bodies, and the spectral responsivity of detectors, based on cryogenic radiometers. Areas and materials being

studied in the Fluorescence Spectroscopy group at BAM include fluorescence standards, fluorometric-screening methods, cation-complexing fluorescent probes, photophysics of dyes, fluorescent sol-gel materials, dye-doped nanocomposites and dye aggregates. BAM has calibrated their fluorometers with PTB-certified spectral radiance and responsivity transfer standards.

Fluorescence measurements with commonly-used dyes, i.e., coumarin 153 and cresyl violet, were performed and demonstrate the existing need for fluorescence standards. The determination of the fluorescence quantum yield of coumarin 153 in ethanol relative to two very well characterized fluorescent dye solutions, quinine sulfate in 0.5 moles/liter sulfuric acid and fluorescein in 0.1 moles/liter NaOH, gave two different values with the larger being about 60 % greater than the smaller. Also, a corrected emission spectrum of cresyl violet in methanol deviated from a published spectrum by more than the uncertainty in the measurement. These inconsistencies may be explained by either difficulties in the proper calibration of the spectral responsivity of the fluorometer's detection system or, less likely, a difference in the purity of the dye used in each experiment.

A questionnaire concerning the need for fluorescence standards was put together at BAM and distributed to participants at the 5th Conference on Methods and Applications of Fluorescence Spectroscopy (MAFS; Berlin, Sept. 1997) and at a NATO workshop on NIR dyes (Trest, Sept. 1997), as well as to selected manufacturers and users of fluorometers in 1998 and 1999. Eighty-two percent and 77 % of those that responded expressed a need for spectral emission standards and quantum yield standards, respectively. Interest for standards with known corrected excitation spectra; i.e., corrected for the wavelength-dependent output of the fluorometer's excitation channel consisting of excitation source, monochromator and other incorporated optical components; and fluorescence lifetime standards was expressed by 66 % and 65 % of respondents, respectively. Interest in polarization standards was least, expressed by 43 % of respondents.

General requirements on ideal luminescence standards include (1) traceability to a primary standard, (2) the report of calibration procedures and instrumental parameters of the reference spectrometer, and (3) specifying uncertainties that have been determined to a sufficient level of statistical confidence. For ideal liquid fluorescence standards, requirements also include (4) high purity, (5) known stability, (6) ready and inexpensive availability, (7) easy handling, (8) a variety available (different spectral regions, Stokes shifts and quantum yields), (9) polarization independence, (10) temperature independence within the range of room temperature, (11) insensitivity to O_2, (12) pH insensitivity and (13) known photophysics.

Spectral emission standards are presently being developed at BAM in cooperation with PTB. One approach includes a dye system where the spectral position of the broad and strongly Stokes-shifted charge-transfer emission band depends on the substituents employed. A separate intramolecular electron transfer process can control the fluorescence quantum yield and lifetime. With this moderate to highly fluorescent dye system, an emission wavelength region from 400 nm to 800 nm can be covered in acetonitrile (solvent), employing three different acceptors. Furthermore, the emission of bipyridyldiol in organic solvents has also been studied. For instance in n-hexane, this stable dye exhibits a very large Stokes shift (9400 cm^{-1}), due to excited state intramolecular double proton transfer and a broad emission band (ca. 450 nm to 650 nm). Its fluorescence quantum yield in this environment is 0.30.

2.10 NRC Reference Spectrofluorimeter and Its Application to the Measurement of Photoluminescent Transfer Standards for Colorimetry

Joanne C. Zwinkels—NRC, Canada

The spectrophotometry metrology activities at the National Research Council of Canada (NRC) are directed towards improving instrumentation and calibration procedures for the accurate measurement of material optical properties. The NRC scales of spectral transmittance [16], spectral reflectance [17] and specular gloss [18] are based on high-accuracy reference instruments. Historically, an important application of these facilities has been color and appearance measurement of non-fluorescent materials. However, in recent years, there have been significant technological improvements in the stability of fluorescent dyes, pigments and inks, which has increased their use in many colorimetric applications. For example, currently many grades of paper and board contain fluorescent whitening agents to enhance their brightness and offset yellowness. It was the demand of the paper industry for international standardization in the measurement of optical properties of fluorescent paper and board that motivated the recent development of a reference spectrofluorimeter at NRC for high-accuracy color measurements of photoluminescent reflecting materials.

The color appearance of an object depends upon the spectral properties of the light source, the light-modifying properties of the object and the spectral sensitivity of the human observer. To give a precise method of color specification, the International Commission on Illumination (CIE) has standardized the illuminant,

measurement geometry and observer conditions. Spectrophotometers that use monochromatic detection for measuring reflectance give erroneous colorimetric results for photoluminescent samples [19]. This is because the color appearance of these materials depends upon the combined subjective effect of the reflected and luminescent radiation. For highest accuracy, these samples require a colorimetric instrument that can control the excitation and detection wavelengths independently. The NRC Reference Spectrofluorimeter fulfills this requirement by employing two separated monochromators, one for monochromatic excitation of the sample and one for monochromatic detection of the reflected and luminescent radiation [20,21]. The instrument has a wavelength range from 250 nm to 1050 nm and illuminates the sample with an angular cone of radiation at an angle of incidence of 45° and detects the radiation reflected/emitted normally to the sample surface. Standard lamps, detectors and a white non-fluorescent reflectance standard are used to calibrate the instrument. A red fluorescent paint sample is used to illustrate the steps involved in measuring the reflected and bispectral luminescent radiance factors and combining these quantities to calculate the total spectral radiance factors and color specification for any desired standard illuminating conditions.

2.11 Traceability, SRMs and NTRMs

John C. Travis—NIST

Quantitative chemical measurements have traditionally been traceable to NIST through certified reference materials (CRMs) whose certified properties are the mass fractions or mole fractions of chemical compounds and/or elements present. Ideally, the small number of such CRMs that can be directly issued by NIST (and designated "Standard Reference Materials" or SRMs) should be leveraged by commercially issued CRMs that rely on SRMs for their traceability link to NIST, while deepening and broadening the availability of materials to end users. The "NIST Traceable Reference Material" (NTRM) program is an effort to formalize such a relationship between NIST and commercial producers of reference materials. Further leverage in promoting chemical measurement accuracy is provided by the recognition that spectrometric measurement quality may be enhanced through "instrument standardization," or the assurance that instruments designed to perform a given spectrometric measurement give consistent results over time and across manufacturers and models. Thus, some of the SRMs supported by the Analytical Chemistry Division of NIST have been certified for such physical properties as absorption feature wavelengths and optical transmittance. The pressures of regulatory and quality system requirements have resulted in an increased demand for appropriate spectrometric reference materials that far exceeds NIST's production capabilities, but can readily be adapted to the NTRM model. Alternative standards to SRMs include inherent standards consisting of readily available materials, traceable commercial artifacts and standard methods from other organizations (ASTM, etc.). In the NTRM model, both the transfer instrument and random NTRMs of producers are periodically certified by NIST. The conversion to the NTRM model for any SRM in large demand should take place as soon as possible from the time that the SRM is first certified.

2.12 Size Dependent Luminescence of Semiconductor Nanocrystals

A. Paul Alivisatos—University of California, Berkeley

Colloidal semiconductor nanocrystals (or nanoparticles) exhibit strongly size-dependent optical properties. These crystals range in diameter from 2 nm to 12 nm, typically, and each one is not identical to another. As a nanocrystal's size increases, the wavelength of its fluorescent emission also increases. This behavior is consistent with particle-in-a-box theory. As the ability to control the size and surfaces of the nanoparticles has developed, the luminescence of these nanocrystals has started to find applications in biological imaging and light-emitting diodes.

A nanoparticle is an intermediate material state between a single molecule with discreet states and a bulk semiconductor. The particle has a threshold excitation energy; i.e., at and above this energy it can be excited; so the excitation energy does not have to be resonant with the molecular transition. This makes it possible for many nanoparticles, each emitting at a different size-dependent wavelength, to be excited at the same wavelength. This is in contrast to commonly used fluorophores, such as organic dyes, that have narrow windows of excitation and nearly fixed Stokes shifts. Also unlike dye emission, nanoparticle emission does not have a long red tail. A bandwidth of about 12 nm and 25 nm is observed here for a single nanoparticle and for the ensemble of nanoparticles, respectively, at room temperature.

Nanoparticles are crystallized in a supersaturated semiconductor solution and are surrounded with detergent so they do not stick together. A group of nanoparticles with a particular size can be made in about 15 minutes, using a method that results in all particles being nearly the same size. Methods requiring size separation are much more time consuming. A homogeneous size distribution, within 5 %, is obtained

with the faster method by regulating the growth rate of the crystals. At relatively large growth rates, rod-shaped, instead of sphere-shaped, nanocrystals are obtained. The rods are of interest because they have polarized emission. Photochemical degradation and nonradiative relaxation are minimized by growing a shell around a core nanocrystal, with the shell semiconductor having a larger bandgap than the core semiconductor. This causes the electron-hole pairs to be confined to the core and not allowed to migrate to the nanoparticle surface.

By adding silica to the core-shell system as a third layer, the nanoparticles are made water-soluble, which is necessary for biological applications. Using two different sizes of CdSe-CdS core-shell nanocrystals enclosed in a silica shell, mouse fibroblast cells have been labeled with green and red fluorescent-nanocrystal probes [22]. Biomolecules labeled with this type of nanocrystal can also retain their biological activity [23]. InAs-CdSe core-shell nanocrystals have been used to explore ways to increase the quantum yields of nanocrystals. As the CdSe shell grows, the quantum yield increases until stress causes the shell to crack, at which point increasing shell size causes the quantum yield to decrease. Quantum yields of nanocrystals must increase further for them to compete with organic dyes as fluorescent probes. Increases in nanocrystal quantum yields continue to be attained, so the forecast in this area is optimistic.

Fluorescence intensity of nanocrystals can change with time, due to intermittent emission. When observing emission from a single nanoparticle, the intensity may be zero for as long as a millisecond. This intermittence becomes more frequent as the excitation intensity is increased. This phenomenon can occur when two electron-hole pairs are created in one nanocrystal. One electron-hole pair transfers its excitation energy to the other in the form of kinetic energy, which enables it to leave the nanocrystal. This results in a nanocrystal with a positive charge and a corresponding electric field. The fluorescence intensity of a particle in an electric field is reduced. At a later time, the nanoparticle will recover, becoming neutral again. Nanocrystals must be perfectly passivated in order for this phenomenon not to occur.

Other phenomena and areas presently being researched include (1) fluorescence from a single nanocrystal does not decay exponentially, implying that there are many ways that it can relax; (2) making outer shells more continuous in core-shell nanocrystals, as photobleaching is then less likely; (3) trying to pattern nanocrystals using DNA, as this could be used to make nano-electronic devices. All of the parameters that determine the photophysics of nanocrystals must be experimentally controlled in the future for such crystals to be used as fluorescence standards.

2.13 Inorganic Complexes as Luminescence Reference Materials

James Demas—University of Virginia

Rare earth and transition metal coordination complexes possess a diverse range of luminescent properties that suggest their use as luminescence standards. Transition metal complexes have shown potential for use as oxygen sensors, quantum counters, spectral emissivity standards and cryogenic thermometers. Rare-earth metal complexes show potential as luminescence lifetime standards. Excitation and emission spectra of d^6 metal complexes are very dependent on the ligands that surround the metal ion, exhibiting changes in wavelength position and luminescence intensity. This is because different coordinating groups around the metal ion change the HOMO-LUMO, highest occupied and lowest unoccupied molecular orbital, spacing [24,25,26]. General characteristics of transition metal complexes include (1) strong, broad absorptions, (2) relatively long lifetimes in solution (100 ns to >100 μs), (3) high luminescence quantum yields (0.01 to >0.7), (4) tunable emission by changing coordinating groups, (5) wavelength-invariant quantum yields, and (6) large Stokes shifts (150 nm to 200 nm).

Ruthenium complexes are quenched by oxygen at room temperature and can be used as oxygen sensors. Stern-Volmer quenching plots for $[Ru(Ph_2phen)_3]^{2+}$; (Ph_2phen) = 4, 7-diphenyl-1, 10-phenanthroline; in silicone rubber show it to be a particularly good oxygen sensor and it is now widely used in commercial systems [24,25,26,27]. $[Ru(bpy)_3]^{2+}$; bpy = 2,2′-bipyridine; can be used as a luminescence quantum counter for measuring the relative intensities of light of different wavelengths [28]. $[Ru(bpy)_3]^{2+}$ in a polyvinyl alcohol (PVA) film has a flatter spectral response from 360 nm to 530 nm than the commonly used methanol Rhodamine B quantum counter solution. A polymer film is also more convenient to use as a quantum counter, because it is not a solution and is less prone to photodecomposition. The fluorescence lifetime of $[Ru(bpy)_3]^{2+}$ in PMMA is strongly temperature dependent from about 2 K to 20 K [29]. This suggests that it could be used as a low temperature non-contact glass thermometer.

A set of three transition metal complex solutions can be used as a spectral emissivity standard, covering a wide wavelength range from 450 nm to 820 nm. This set is comprised of $Ir(bpy)_2Cl_2^+$, $[Ru(bpy)_3]^{2+}$ and $[Os(phen)_3]^{2+}$; phen = 1, 10-phenanthroline; which emit in blue-green, orange and red spectral regions, respectively. Only the ruthenium complex is commercially available. The osmium complex luminesces the most weakly. The luminescence lifetime of $[Eu(dpa)_3]^{3-}$, dpa = dipicolinic acid, in water is about 1.6 ms at room

temperature. This solution is a potential lifetime standard in the millisecond regime as it is very stable and weakly temperature dependent [30].

2.14 Luminescence Standards for Quantitative Fluorescence Cytometry

Robert F. Vogt, Jr.—CDC

Fluorescence cytometry (FC) is a powerful technique for characterizing genetic and proteomic markers of cells and is employed in many areas of biomedical research and clinical diagnostics. Despite increasing clinical use, FC is not yet standardized in its quantitative measurement of fluorescence intensity. The calibration of fluorescence intensity is essential for FC to quantify differing degrees of expression in living cells. The increased reliability in these measurements through routine calibration will improve the diagnostic value of FC and thereby enhance patient care. Fluorescence intensity standardization would be especially valuable for longitudinal population-based studies, such as clinical drug trials and public health studies, since predicting value is highly dependent on consistent data collection and retrospective evaluation.

The Public Health Service (PHS), which includes the Centers for Disease Control and Prevention (CDC), Food and Drug Administration (FDA) and National Institutes of Health (NIH), advises NIST in biomedical issues. Collaborations between PHS and NIST help to link biochemical research with commercial applications. One such collaboration presently in progress involves the development of a fluorescein concentration SRM, which will be used to calibrate particulate standards for FC and other solid-phase applications, including gene chips.

Standards that are needed in quantitative FC include (1) physical standards, (2) procedural standards, (3) nomenclature standards and (4) application standards. Physical standards are needed in the forms of fluorescent solutions and fluorophore-labeled particle suspensions for cuvette, flow and scanning platforms. The purpose of these standards is to establish proportionalities between fluorescence intensity and the number of cells present within the illuminated volume. A commonly used way to express this proportionality is in MESF, molecules of equivalent soluble fluorochrome "units." For example, if 10 cells suspended in a given volume of buffer fluoresce with the same intensity as a solution of fluorescein containing 10^6 molecules in the same volume of buffer, both measurements being taken on the same instrument under the same conditions, then each cell in the suspension is said to fluoresce at 10^5 MESF units. A. Schwartz introduced MESF units to the FC community and founded Flow Cytometry Standards Corp., San Juan, PR. They have sold fluorescently labeled microbeads with specified MESF units ranging from 5×10^3 to 10^6. Non-fluorescent microbeads give background values of a few hundred MESF.

Fluorescein isothiocyanate (FITC) is the most commonly used fluorescent label for cells and biological molecules. Different binding environments can have a significant effect on the fluorescence spectrum of FITC, so it is important to establish procedural standards to define MESF units for different systems. NCCLS is a standards organization, formerly known as the National Committee for Clinical Laboratory Standards, with concerns in this area. Procedural and nomenclature standards for quantitative FC are being established by NCCLS through a consensus process by the subcommittee on Fluorescence Calibration and Quantitative Measurements of Fluorescence Intensity. The guidelines proposed by this subcommittee are due in 2001. Proposed nomenclature standards for FC include QFCM, quantitative fluorescence (or flow) cytometry (or cytometric measurement), and ABC, antibody binding capacity. ABC determination by QFCM is the preferred method for measuring the expression of surface receptors on cells.

2.15 Fluorescein Fluorescence Intensity SRM

Adolfas K. Gaigalas—NIST

The clinical, medical and biotechnology communities have a pressing need for fluorescence intensity standards to calibrate flow cytometers and/or microscope imaging/scanning systems. At two recently held, well-attended NIST workshops on the subject, leaders from these fields called upon NIST to develop a fluorescence intensity standard based on fluorescein. This material was chosen for the initial standard because it is available in reasonably pure form, has been well studied as a fluorescent material and is representative of the commonly used fluorescent label FITC and other fluorescein-labeled tools such as microspheres, antibodies and derivatizing agents.

The fluorescein SRM will be certified for concentration. It will be a concentrated ($\approx$60 μmol/L) solution of fluorescein in 0.1 moles/L borate buffer at pH 9.0. To avoid the inherent difficulties of using volumetric units, the reference solution will be calibrated in units of g of fluorescein per g of buffer. The reference solution can be used to determine the concentration of fluorescein in arbitrary buffers. In practice the reference solution will be diluted, by say 100-, 200-, . . . fold, using the same buffer in which the unknown fluorescein is found. The diluted reference solutions will provide a calibration of fluorescence intensity as a function of fluorescein concentration. These intensities can be compared with

other solutions of fluorescein or its derivatives in other environments to determine the MESF units of these solutions.

Before this SRM is certified, the extinction coefficient, photostability and purity of the fluorescein being used will be measured. The SRM is expected to be on the market by autumn of 2000. Methods are also being developed in the NIST Biotechnology Division for producing and characterizing a fluorescein-immobilized-on-a-microbead SRM, with certified MESF values relative to fluorescein. These values will be certified on a Reference Fluorescence Flow Cytometer at NIST.

2.16 Relative Raman Intensity Calibration Through Fluorescent Glass Standards

Steven Choquette—NIST

A Raman spectrum is a convolution of the spectrum of the sample and the instrument response function. Ohio State University professor R. L. McCreery has proposed a procedure employing fluorescent standards with known emission curves to correct the measured Raman spectroscopic intensity. This is an important step toward creating standardized Raman spectral libraries. At present, no standard Raman libraries are used because Raman intensities are instrument dependent. Like IR spectroscopy, Raman spectroscopy is commonly used for qualitative chemical analysis. Libraries will be necessary if Raman is to be used in quantitative applications requiring adherence to regulatory standards. Lack of performance validation standards inhibits the industrial use of Raman, as two different instruments can not obtain the same spectrum without them. NIST has developed a number of stable, fluorescing glasses that will be evaluated as secondary spectral emissivity standards for the relative intensity correction of Raman spectra.

Absolute intensity calibration of a Raman spectrometer can be performed using black body emitters, calibrated white light sources or fluorescent standards. Of the three, fluorescent standards are the least expensive by far. Also, black body emitters and white light sources are difficult to align and fail to reproduce the Raman emission geometry. An ideal glass fluorescence standard should (1) have a broad, featureless spectrum over the relevant wavelength range, (2) reproduce the Raman emission geometry, (3) be homogeneous, photostable and have a long shelf-life, (4) provide reproducible fluorescence intensity and shape and (5) have a matrix with the flexibility to allow doping of fluorescent materials with common laser excitation wavelengths. Commonly used laser wavelengths are 514 nm (Ar-ion laser), 532 nm (frequency-doubled Nd:YAG laser), 780 nm and 785 nm (diode lasers) and 1064 nm (Nd:YAG laser).

The three fluorescent glasses currently being evaluated as Raman emission standards contain U, Cr, and Eu oxides for 514 nm/532 nm, 780 nm/785 nm and 1064 nm excitation, respectively. By increasing the concentration of dopant, these glasses are potentially viable as fluorescence standards.

3. Discussion

During the open discussion session that closed out each day, the search for consensus on those fluorescence standards that are most needed revealed several standards in demand. The greatest interest was expressed for the development of instrument validation standards for fluorometers, particularly for the clinical and biotechnological communities. These groups need to have standards that establish proportionalities between concentration or amount of a target species (cells, proteins, DNA, etc.) and fluorescence intensity. Spectral emissivity standards, with long-term stability and covering a broad wavelength region (400 nm to 1000 nm), are also needed by these communities, as well as the general fluorescence community. A wavelength standard that has sharp peaks in the green to red regions of the spectrum (500 nm to 700 nm) is also in demand. Inorganic fluorophores have many desirable characteristics, but many in the biological community seem reluctant to use materials that are not organic-fluorophore-based. For more experienced fluorescence researchers, standards with certified quantum yields and extinction coefficients are desirable.

Continued communications between workshop attendees was mutually supported and all attendees will receive a copy of this workshop summary. Correspondence, focused on attaining more extensive collaborations and future CRADAs, is being established between particular attendees and NIST. The prospect of creation of a website to provide up-to-date information concerning fluorescence standards activities at NIST was also met with great encouragement.

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